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Inactivation of Glyceraldehyde 3-Phosphate Dehydrogenase

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Enzymatic activity and physical chemical properties have been determined for yeast glyceraldehyde 3-phosphate dehydrogenase (GPD) exposed to low and high pH. Inactivation of the enzyme is rapid below pH 4.5 and above pH 11, and is accompanied by a decrease in sedimentation constant resulting in large part from dissociation into its constituent subunits. Both the sedimentation and optical rotation dispersion behavior of inactivated GPD indicates that a drastic unfolding of the subun it has probably occurred. The ORD parameters of native yeast GPD are comparable to those previously reported for the apo-muscle enzyme and suggest a similarity in their secondary and tertiary structures. The relative magnitude of these parameters suggests that GPD contains ordered structural elements other than helix and random cha n. The drastic changes for a_0 and b_0 observed in the regions of inactivation of yeast GPiD appear to be due largely to "melting out" of these nonhelical regions. Acid inactivation appears to give as disordered a structure as is observed on urea denaturation, while the process at high pH appears to be less complete.

Harris and Perham (1) have demonstrated that the chemical subunits of glyceraldehyde 3-phosphate dehydrogenase (GPD)[2] are identical polypeptide chains. Molecular weight studies of Dandliker and Fox (2, 3), and more recently by Harrington and Karr (4), indicate that four of these subunits most probably make up the quaternary structure of the native enzyme. The near identity of the sedimentation constants for the yeast and muscle enzymes (5) suggests that both enzymes have comparable molecular weights. Amino acid and end group analysis and peptide mapping likewise indicate that the yeast enzyme consists of four identical peptide chains (1). Deal (6, 7) has reported that both yeast and rabbit muscle GPD could be dissociated into subunits of very low molecular weight through the action of urea or of sodium dodecyl sulfate. Harrington and Karr (4) have shown quite recently that exposure of muscle GPD to 5 M guanidine-HCl results in dissociation into subunits of 35,000 (rabbit muscle) and 36,500 (pig muscle). The results to be reported here indicate that inactivation of yeast GPD which occurs at pH's of below 4.5 and above 11, results in major structural alterations including dissociation into subunits. The ORD parameters obtained for the native yeast enzyme suggest a close similarity of the tertiary and quaternary structures for the yeast and muscle enzymes. They further indicate that the native enzymes contain structural elements other than helix and random chain.

EXPERIMENTAL

Yeast GPD was prepared from starch free baker's yeast by the method of Krebs (8). EDTA (10^{-3} M) was present during all steps of the preparation. Homogeneity of GPD preparations was established using ultracentrifugation (pH 6.6–9.5) and starch-gel electrophoresis (pH 8.6, 0.01 M Tris, veronal or phosphate buffers). GPD preparations were stored at 1°C as crystalline suspensions in

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[2] Abbreviations used: Glyceraldehyde 3-phosphate dehydrogenase—GPD; Optical rotation dispersion—ORD.

0.8-saturated ammonium sulfate. Enzymatic assays were carried out in the presence of 5×10^{-3} M cysteine and 10^{-3} M EDTA according to the method of Krebs (8) using glyceraldehyde 3-phosphate as a substrate. Specific activity of our GPD preparations were typically 50 to 70% of values reported by Warburg and Christian (9) and had A_{280}/A_{260} ratios of about 1.75, indicating the binding of less than 0.3 mole of DPN per mole of GPD (10). Concentrations of GPD were determined by ultraviolet analysis using an extinction coefficient of $E^{1\%}_{280}$ of 9.12. The latter value was determined in a pH-8 ammonium formate buffer by measurement of extinction and drying of aliquots to constant weight over NaOH and P_2O_5. This value is in good agreement with the value of 9.08 reported earlier (11). Reagent-grade chemicals and glass-distilled water were used throughout.

Solutions of enzyme were prepared by dissolving the appropriate amount of the GPD suspension (see above) in 0.015 M KCl containing 10^{-3} M EDTA and then dialyzing against numerous changes of the same solvent for about 24 hours. The resulting ammonium sulfate-free solution maintained at 2°C was adjusted to the desired pH by the careful addition of very small increments of 1 M KOH or HCl from an ultramicro buret with efficient stirring. Measurement of pH was made with a Radiometer Model 4 pH meter.

Sedimentation velocity measurements were made in double-sector cells in the Model E ultracentrifuge at a speed of 50,740 rpm. The temperature was regulated at 4° during sedimentation experiments. Sedimentation constants were corrected to 20° using appropriate values of solvent density and viscosity. A partial specific volume of 0.735 was used.

ORD measurements were made with a Rudolph Model MSP-4 double monochromator manual spectropolarimeter. The procedures employed have been described previously (12, 13). Data for Moffitt-Yang analysis (14) were obtained with mercury lines (313, 334, 365, 405, 436, 547, and 578 mμ) from a low-pressure lamp. ORD data below 300 mμ was obtained with a 450-W xenon light source. The temperature was maintained at 2° by circulating constant temperature water through the cell jackets. Residue rotations were calculated using a value of 108 for the mean residue weight as obtained from the amino acid composition (5). Refractive index data used in this computation were interpolated from values tabulated for water (International Critical Tables) using the Sellmeier formula.

RESULTS

Inactivation of GPD. In order to determine the pH range of stability of GPD, solutions of the enzyme in 0.015 M KCl at concentrations of 0.5 to 0.7 gm/100 ml were titrated to the desired pH by careful addition of 1 M HCl or 1 M KOH. In many instances the identical solutions were used for ultracentrifuge and ORD experiments (see subsequent sections). At predetermined periods, aliquots were removed and diluted 100-fold with a pH 8.2 "stopping" buffer (10^{-3} M EDTA, 5×10^{-3} M, Tris, 0.15 M KCl). Assays were carried out within 30 minutes of dilution. Since we have found that reactivation of acid or alkaline denatured GPD is not readily accomplished (see below), the time between "stopping" and assay is not critical. Representative inactivation data are shown in Fig. 1. Additional points obtained at 200 minutes have been omitted in the interests of clarity. GPD is relatively stable at pH values as low as 4.5. Below this pH, however, the rate of inactivation is very markedly pH dependent, e.g., at pH 4.25 about 70% of the activity is retained after 400 minutes, while for a comparable time at pH 4.10, only about 4% of the original activity remained. Inactivation at alkaline pH values occurs above 11, although the process appears to be somewhat less sensitive to pH than that observed in the acid range, activity still being evident at pH 11.5 after 400 minutes. Stockell (15) has previously noted the inactivation of yeast GPD below pH 4.5 and above 11.

Attempts to reactivate acid or alkaline denatured GPD were uniformly unsuccessful regardless of the procedures employed. These included rapid titration to pH 8, dialysis against pH 8 buffers in the presence and absence of DPN and cysteine. In a few experiments some reactivation (5–15% of the original) was obtained in the presence of 0.1 M mercaptoethanol. It should be emphasized that these reactivation experiments refer to GPD exposed to acid or alkaline pH's for 25 minutes or more. No attempt was made to study the effect of shorter periods of exposure of GPD to extremes of pH, since our primary aim in this study was to relate changes in activity to alterations in molecular parameters which could only be obtained in relatively long experiments.

ORD properties. In the pH range where GPD remains relatively active (see Fig. 1), the ORD parameters showed little time

dependence; for example, at pH 5.35, a_0 increased in levorotation by only 5% in about 30 hours while b_0 remained virtually unchanged (see Figs. 2 and 3). These parameters at pH 4.25 show changes of about 10–20% after about 30–60 minutes, indicating a certain amount of structural alteration. This should be compared with a loss of about 30% of the original activity after comparable time (see Fig. 1). The rate of change of the ORD parameters, like that of the loss of activity, increases rather abruptly below pH 4.25. At pH 4.10, for example, a_0 increased in levorotation by nearly twofold in about one hour, while b_0 had decreased to about 50% of the value observed for the native protein (Fig. 1) Comparable time dependencies of the ORD parameters were noted above pH 11, although as we shall see subsequently, the values of a_0 ultimately attained were significantly lower.

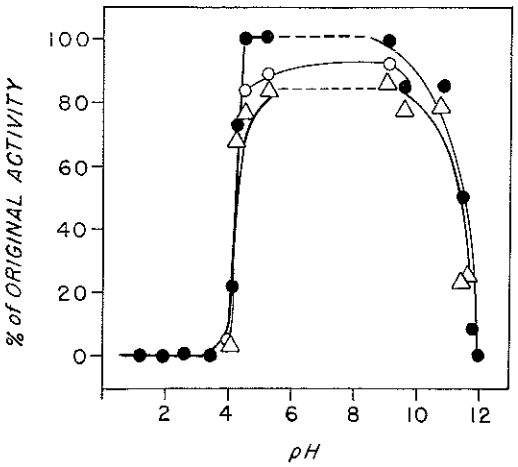

FIG. 1. Inactivation at 2° of yeast GPD at acid and alkaline pH. Minutes of exposure to the pH indicated: ●—25 minutes; ○—200 minutes; △—400 minutes. See text for procedure.

Shown in Figs. 4 and 5 are the pH dependencies of a_0 and b_0 obtained 30 minutes to 1 hour after adjustment to the pH indicated. About 30 minutes was required for the completion of an ORD curve at each pH. In the range where time dependencies are observed (*ca.* 3.4–4.5 and 11.2–11.6), the values are subject to some uncertainty. As indicated above, marked changes in a_0 and b_0 become apparent just above pH 4 (compare also with Fig. 1 and ORD data above pH 11). Below pH 3, b_0 attained a value of about −30° as compared to −130 to 135° observed with the native protein, while a_0 increased in levorotation from about −240° to nearly −600°. Shown also for comparison (dot-dashed lines) are the values of a_0 and b_0 observed for GPD denatured in 8 M urea (pH 8.6, 0.05 M pyrophosphate buffer). Of particular interest is the fact that the a_0 value observed below pH 3 approaches that found for the urea-denatured enzyme. The value of

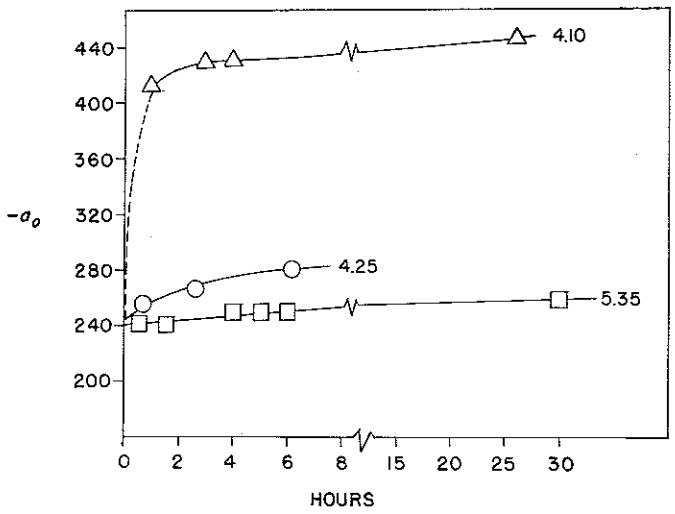

FIG. 2. Time dependence of ORD parameter, a_0, for yeast GPD at 2°. Protein conc., 0.5–0.7 gm/100 ml; solvent, 0.015 M KCl; △—pH 4.10; ○—pH 4.25; □—pH 5.35.

b_0 for acid-denatured GPD however, is about 20 to 30° more levorotatory than the value found for urea-denatured enzyme. In view of the poor precision of determination of low values of b_0, this difference may not be significant.

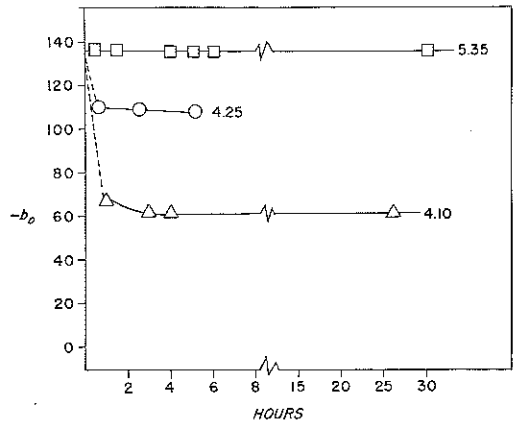

FIG. 3. Time dependence of ORD parameter, b_0, for yeast GPD. Conditions and symbols as in Fig. 2.

Values of b_0 obtained near neutral pH were about −135°, in good agreement with Havsteen's (16) value of −131 ± 6° for the yeast enzyme at pH 7.7. Since our principal concern in this study was the behavior of GPD at high and low pH, data taken in the pH range 7 to 9 is probably too sparse to confirm his observation of an increase in levorotation of b_0 of about 20° in going from pH 7.7 to 8.4. Moffitt–Yang plots of the ORD data were linear within experimental error over the wavelength range 313 to 578 mμ in contrast with the observations of Havsteen who found anomalous dispersion effects near 305 and 650 mμ.

At high pH, b_0 was found to be comparable to that observed with acid-denatured GPD but the highest value of a_0 attained is 100° more levorotatory (Fig. 4). Measurements of the ORD parameters as a function of time gave no indication that a_0 at high pH would approach the value observed at low pH. At pH 11.96, for example, no significant time dependence of rotation was noted from 0.5

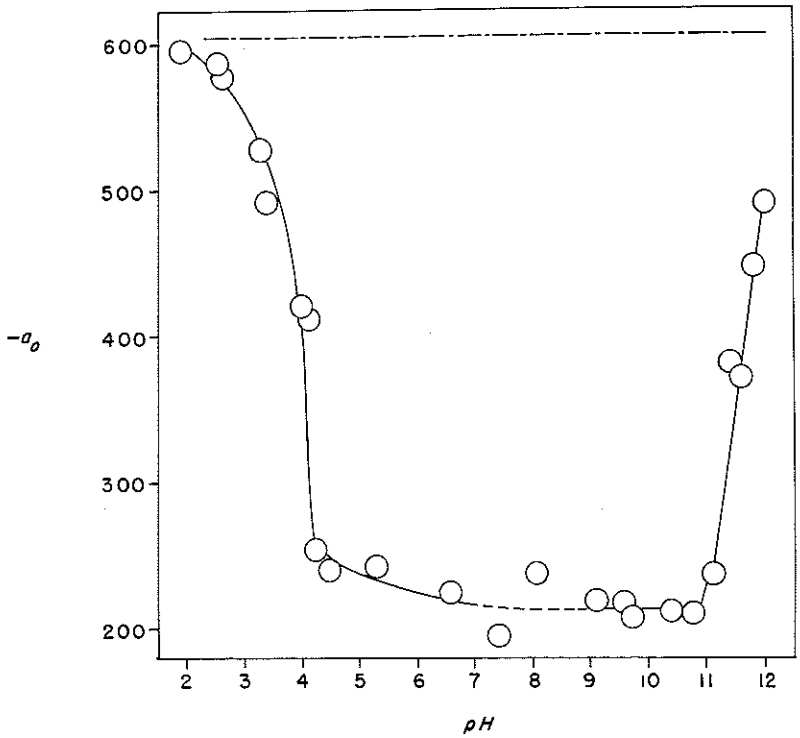

FIG. 4. pH dependence of a_0 for yeast GPD. Conditions as in Fig. 2. Time of measurement, 0.5–1 hour after pH adjustment. (-·-·-) value observed after denaturation in 8 M urea at pH 8.6.

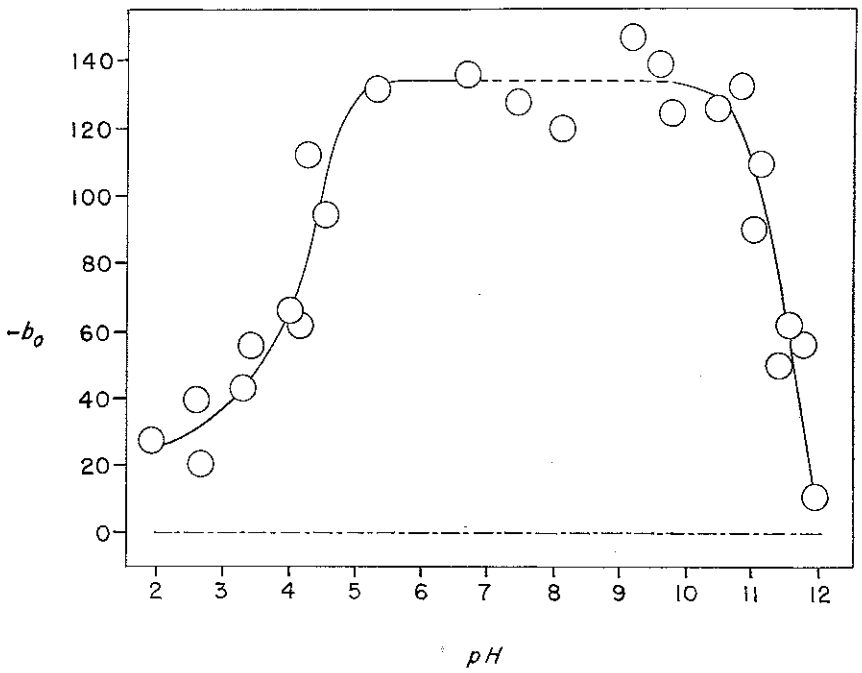

FIG. 5. pH dependence of b_0 for yeast GPD. Conditions and symbols as in Fig. 4.

to 5 hours, and the corresponding value of a_0 was only about $-500°$. This difference in a_0 appears to be a reflection of a genuine difference in the nature of the structural alteration at high and low pH which is also borne out by sedimentation experiments (see subsequent section). At pH 11.55, while both a_0 and b_0 were time dependent up to about 7 hours, little or no change in the ORD parameters were noted for the succeeding 16 hours, with a_0 attaining a maximum value of about $-450°$.

The ORD characteristics of native and acid-denatured GPD (Fig. 6) appears to be quite ordinary in the wavelength range below 300 mμ. There appears to be little sign of side-chain Cotton effects in the 260 to 270 mμ region, although the relative flatness of the curve for the pH 7 enzyme in the range 275 to 285 mμ may be indicative of a very weak one. This observation is comparable with Listowski *et al.* (18) observation of a weak inflection in the ORD curve for the native apo-muscle enzyme. ORD curves for native and acid-denatured GPD exhibit a trough at about 233 mμ with amplitudes of -5000 and $-4000°$, respectively.

The reduction in the amplitude of the 233-mμ trough occurring on acid denaturation is consistent with a "melting out" of helical regions of the GPD molecule (17). However,

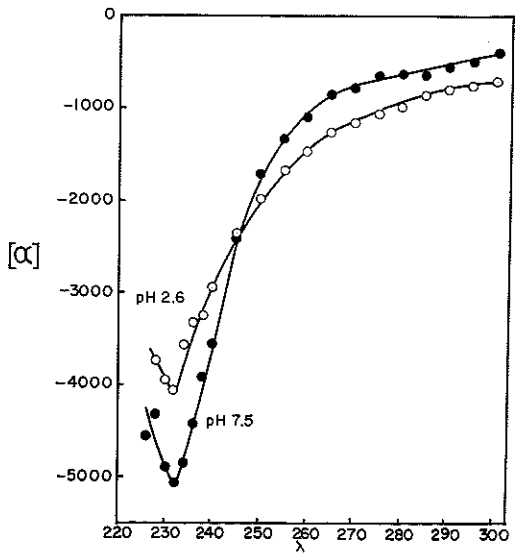

FIG. 6. Optical rotation dispersion of yeast GPD in the wavelength range 225 to 300 mμ. Protein concentration, 0.05–0.15 gm/100 ml. Other conditions as in Fig. 4.

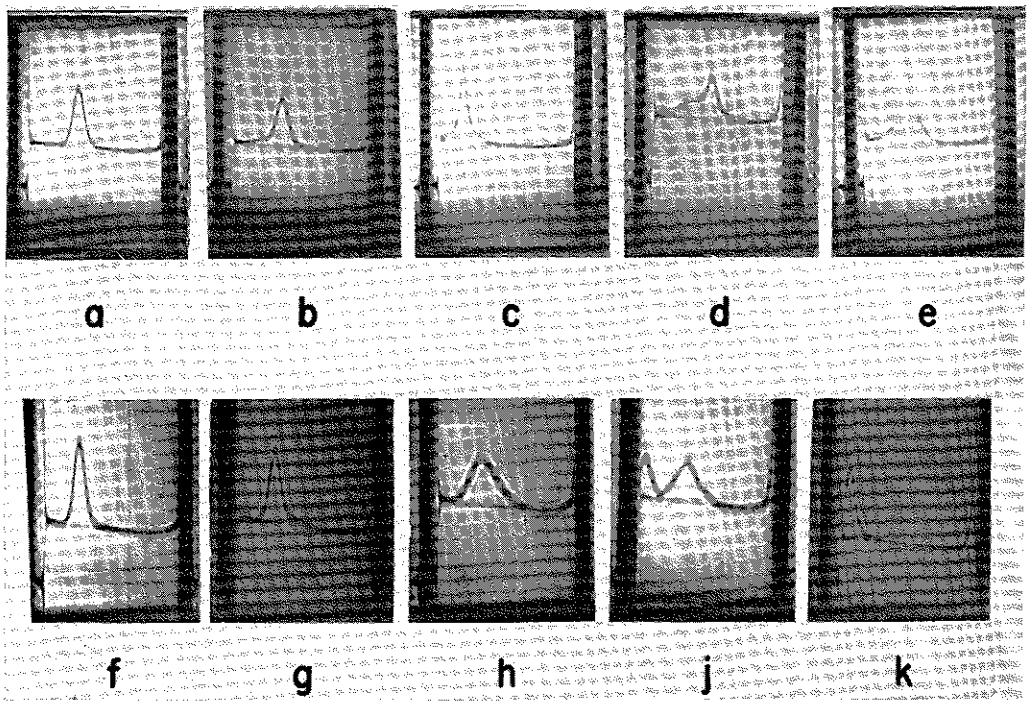

FIG. 7. Sedimentation patterns of yeast GDP at various pH values. Speed, 50,740 rpm; protein conc., 0.5–0.7 gm/100 ml; temperature, 4°; solvent, 0.05 M KCl; all photographs shown are for 96 minutes of centrifugation with the exception of d and e which were at 336 minutes. a—pH 9.71; $S_{20,w}$, 7.27; b—pH 10.80; $S_{20,w}$, 7.32; c—pH 11.40; $S_{20,w}$, 3.50; d—pH 11.75; $S_{20,w}$, 2.1, 1-1.5; e—pH 11.96; $S_{20,w}$ 2.5, 1.6; f—pH 5.26; $S_{20,w}$, 7.59; g—pH 4.25; $S_{20,w}$, 7.50; h—pH 4.01; $S_{20,w}$, 8.07; j—pH 3.40; $S_{20,w}$, 10.1, 1.3; k—pH 2.66; $S_{20,w}$, 1.1.

as we shall consider in the Discussion Section, the ORD properties of the native enzyme do not appear to be consistent with a structure composed solely of helical and random-chain regions.

Sedimentation properties. Examination of GPD in the ultracentrifuge indicated that the active enzyme (pH 4.5 to 11) sedimented as a single boundary (Figs. 7 and 8) with a sedimentation constant of 7.3 to 7.5S in good agreement with the value of 7.71 (infinite dilution) reported by Fox and Dandliker (2, 3) for the muscle enzyme, and 7.47 (infinite dilution) obtained by Allison and Kaplan (5) for the yeast enzyme.

The pH dependence of the sedimentation constant of GPD showed abrupt changes comparable to those observed in ORD and activity. Below pH 4 and above pH 11, drastic decreases in $S_{20,w}$ occur. At pH 11.40, a single boundary (3.5S) was observed, while at still higher pH values, bimodal patterns were evident ($S_{20,w}$, 2 to 2.5 and 1 to 1.5). Below pH 4, slowly sedimenting components (1 to 1.5S) were observed. The magnitude of these sedimentation constants, as well as those observed at high pH values are comparable to the value of 1.83S observed by Harrington and Karr for the muscle GPD subunit. It seems probable, therefore, that the inactivation process occurring about 11 and below pH 4.25 involves, at least in part, dissociation into subunits.

While GPD sediments as a single slow boundary below pH 3, biomodal patterns are observed between pH 3 and 4. The faster of these components has a sedimentation constant greater than that of the native enzyme (see pH 3.40; Fig. 7, for example). This heavier component appears to be a nonspecific aggregate of dissociated subunits. Prolonged exposure of GPD to pH 3.40, e.g.,

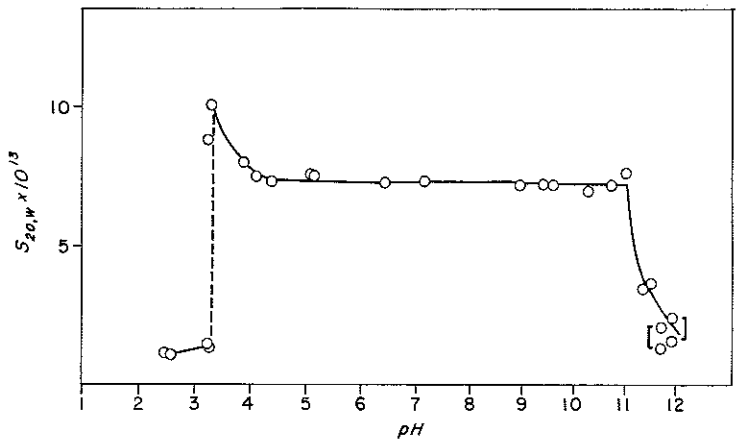

FIG. 8. pH dependence of the sedimentation constants of yeast GPD. Conditions as in Fig. 7. Brackets indicate bimodal patterns for a given experiment.

24 hours, increased the amount of the 10.1S component at the expense of the 1.34S component. Similarly, extended exposure of GPD to still lower pH values in some cases produced components as heavy as 19S. It seems probable, therefore, particularly in view of the changes in activity (Fig. 1) and in ORD properties (Figs. 4 and 5), that the 8.07S component observed at pH 4 is a nonspecific aggregate of subunits rather than the native molecule.

DISCUSSION

ORD properties of native GPD. A comparison of the ORD properties of yeast and muscle GPD indicates a close similarity in the tertiary and quaternary structure of the two enzymes. Values for the latter enzyme were taken from studies of Listowski *et al.* (18). Values of a_0, b_0 and $[m']_{233}$ for the yeast and the DPN-free muscle enzyme differ by 5 to 7% (compare lines 1 and 5, Table I).

The amplitude of the 233-mμ trough (Fig. 6) and the magnitude of the parameters a_0 and b_0 should be a measure of the fraction of the peptide backbone of GPD in the helical conformation provided that the only structures found in the enzyme are helix and random chain. A test of the latter requirement can be made by comparing apparent helical contents calculated from the parameters A_{193} and A_{225} derived from the ORD data (19):

$$F_{225} = -(A_{225} + 60)/19.9, \quad (1)$$

$$F_{193} = (A_{193} + 750)/36.5. \quad (2)$$

Alternatively, the apparent helix content calculated for a_0 and b_0 may be compared (20):

$$F_{a_0} = [(a_0)_{obs} - a^R{}_0]/a^H{}_0, \quad (3)$$

$$F_{b_0} = (b_0)_{obs}/b^H{}_0, \quad (4)$$

where $a^R{}_0$ is the intrinsic residue rotation and $b^H{}_0$ and $a^H{}_0$ are the contributions of helix to the rotation. For $\lambda_0 = 212$ mμ, $b^H{}_0$ is $-630°$ and $a^H{}_0$ is about $+680$ (20). Shown in the first line of Table I are the ORD parameters and the derived values of F obtained with native yeast GPD. The value of $-604°$ used for $a^R{}_0$ in Eq. (3) corresponds to the observed value of a_0 for urea-denatured GPD (line 3, Table I). Values of F calculated for native GPD from a_0 and b_0 differ by more than a factor of 2 (line 1, Table I). The apparent fractions of helix calculated from A_{225} and A_{193} likewise differ significantly. Similar comparisons made of F_{a_0} and F_{b_0} for apo-muscle GPD indicate a comparable discrepancy in helix fraction (line 5, Table I). It would thus appear that both native yeast and muscle GPD contain structural elements other than helix and random chain.

By contrast with the ORD properties of β lactoglobulin, a protein which also appears to contain structural elements other than helix and random chain (21), GPD exhibits

TABLE I

ORD PROPERTIES OF GPD AT 2°

GPD	$-a_0$	$-b_0$	$-[m]_{233}$	$-A_{226}$	$-A_{193}$	Apparent fraction of helix, F			
						a_0[f]	b_0[g]	A_{225}[h]	A_{193}[h]
1. Native yeast[a]	228	136	4120	528	−436	0.55	0.22	0.24	0.33
2. Alkaline-denatured yeast[b]	489	11	—	240	280	0.17	0.02	0.09	0.13
3. Acid-denatured yeast[c]	586	39	3290	347	262	0.03	0.06	0.14	0.13
4. Urea-denatured yeast[d]	604	0	—	240	430	0	0	0.09	0.09
5. Native apo-muscle[e]	245	145	3900	—	—	0.53	0.23	—	—

[a] pH 6.63 for Moffitt–Yang parameters; pH 7.50 for $[m]_{233}$.
[b] pH 12.
[c] pH 2.6.
[d] pH 8.6, 8 M urea.
[e] Data taken from work of Listowski *et al.* (18).
[f] Calculated according to Eq. (3).
[g] Calculated according to Eq. (4).
[h] Calculated according to Eqs. (1) and (2).

but a single trough at 233 mμ (Fig. 6). β lactoglobulin, which yields an infrared spectrum containing bands normally associated with β structures, exhibits a bimodal ORD trough between 228 and 240 mμ (21). Whether these differences in the ORD properties of GPD and β lactoglobulin are due to different "unusual" structural elements remains unanswerable at present. It is tempting to postulate that the "unusual" structural elements in GPD are interchain β conformations formed by hydrogen bonding between dissociable subunits. In view of our limited knowledge of the ORD properties of such structures, however, such a conclusion seems unwarranted at present.

Acid and alkaline inactivation of GPD. The sharp decrease of the sedimentation constant of GPD at both low and high pH (Fig. 8) indicates that inactivation occurring under these conditions is accompanied by dissociation into subunits. Both sedimentation and ORD experiments suggest however, that the two processes are not identical. At acid pH, aside from the presence of high-molecular-weight aggregates, only a single component of sedimentation constant, 1 to 1.5S, is observed. By contrast, a single 3.50S component is seen at pH 11.4 while at still higher pH, a 2 to 2.5S component is observed in addition to the 1 to 1.5S component. The magnitude of the sedimentation constants for the slower component is consistent with drastically unfolded subunits of about 35,000 molecular weight since a compact molecule of such a molecular weight would have a sedimentation constant of the order of 3S. The faster components observed at high pH, therefore, must be either partially dissociated GPD molecules or incompletely disordered but completely dissociated subunits. Our data does not enable us to distinguish between these two possibilities.

The relative magnitudes of the ORD parameters observed with the acid- and alkaline-denatured GPD likewise indicates that molecular disruption is less drastic under the latter condition. At low pH, the ORD parameters approach those observed in urea, while at high pH the highest value attained by a_0 is about 100° lower. This difference in ORD properties of acid and alkaline-denatured GPD is strikingly illustrated in Fig. 9. Each point represents a value of a_0 and b_0 obtained at a given pH at a given time. The data for alkaline-denatured GPD falls on a different curve than that for the acid-denatured enzyme. Also shown is a curve calculated from Eqs. (3) and (4) for a hypothetical structure composed of only helix- and random-chain regions. As indicated in the previous section, the relatively low value of a_0 observed for the native enyzme is most likely indicative of structures other than helix and random chain. Acid-denaturation appears to involve "melting

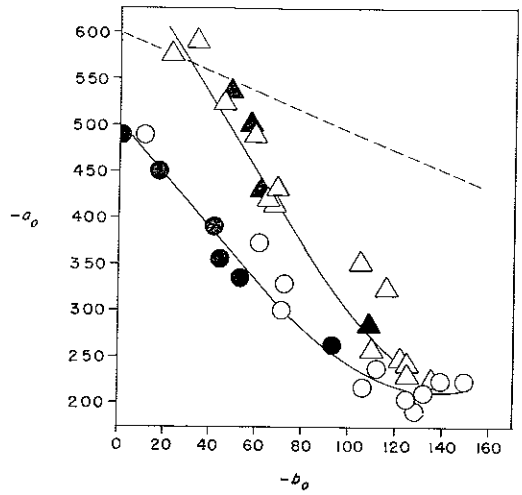

FIG. 9. Comparison of a_0 and b_0 for acid- and alkaline-inactivated yeast GPD. Experimental conditions as in Fig. 4. The figures represent corresponding values of a_0 and b_0 observed at a given pH at a given time. (○, ●)—alkaline-inactivated enzyme (pH 9–12); (△, ▲)—acid-inactivated enzyme (pH 2–5). Unfilled symbols, data obtained 30–60 minutes after pH adjustment. Filled symbols, data obtained 4–6 hours after pH adjustment. (– – –)—curve calculated for a helix–random chain model (see text).

out" of these "aberrant" structures to yield essentially a random-chain configuration. The alkaline-denatured enzyme however, appears to retain some of this nonhelical structure in spite of the fact that, in terms of a helix–random-chain model, the b_0 value would indicate complete disruption.

A comparison of the pH dependence of the parameters a_0 and b_0 for yeast and muscle GPD is of interest. For holo-muscle GPD, a_0 shows a monotonic increase in levorotation in going from pH 7.5 to 10, while b_0 exhibits a monotonic decrease (18). These observations should be contrasted with the relative constancy of these parameters for the yeast enzyme over the pH range 5 to 11 (Figs. 4 and 5). While the values of a_0 and b_0 for the muscle enzyme at pH 10 are roughly comparable to those obtained with the yeast enzyme at the same pH, the values at pH 3 are markedly different; b_0 for the yeast enzyme is about 100° less levorotatory, while a_0 is more than 200° more levorotatory than the muscle enzyme [compare the data of Figs. 4 and 5 with (18)].

The decrease in ORD parameters for the muscle enzyme with change in pH appears to be due largely to dissociation of DPN from the enzyme. While the pH dependence of the binding of DPN to the muscle enzyme does not appear to have been studied, Stockell (15) has shown that the affinity of the yeast enzyme for DPN decreases monotonically below pH 6 and above pH 7. Furthermore, Listowski *et al.* (18) have demstrated for the muscle enzyme that a_0 and b_0 depend markedly on the extent of binding of DPN. The differences in ORD parameters for the yeast and muscle enzymes suggest that no major structural change occurs in the latter case at pH 3. This conclusion is lent support by the apparent absence of time-dependent changes in ORD parameters for the muscle enzyme, as well as the constancy of the amplitude of the 233-mμ trough with change in pH (18). The latter observation should be contrasted with our findings for the yeast enzyme (see Fig. 6). The apparent stability of the mammalian as compared to the yeast enzyme may be due (1) to inherent structural differences of the two enzymes, or perhaps (2) to stabilization by DPN which might still be bound to the muscle enzyme at low pH. The greater thermal lability of the apo-enzyme as compared to the native muscle enzyme (22) would be in accord with the second hypothesis.

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